RESEARCH ARTICLE

Gene expression profiling of nonhuman primates exposed to aerosolized Venezuelan equine encephalitis virus

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Abstract

Host responses to Venezuelan equine encephalitis viruses (VEEV) were studied in cynomolgus macaques after aerosol exposure to the epizootic virus. Changes in global gene expression were assessed for the brain, lungs, and spleen. In the brain, major histocompatibility complex (MHC) class I transcripts were induced, while the expression of *S100b*, a factor associated with brain injury, was inhibited, as was expression of the encephalitogenic gene *MOG*. Cytokine-mediated signals were affected by infection, including those involving IFN-mediated antiviral activity (*IRF-7*, *OAS*, and *Mx* transcripts), and the increased transcription of caspases. Induction of a few immunologically relevant genes (e.g. *IFITM1* and *STAT1*) was common to all tested tissues. Herein, both tissue-specific and nontissue specific transcriptional changes in response to VEEV are described, including induction of IFN-regulated transcripts and cytokine-induced apoptotic factors, in addition to cellular factors in the brain that may be descriptive of the health status of the brain during the infectious process. Altogether, this work provides novel information on common and tissue-specific host responses against VEEV in a nonhuman primate model of aerosol exposure.

Introduction

Venezuelan equine encephalitis viruses (VEEV) are small, positive-stranded RNA viruses in the genus *Alphavirus* of the family *Togaviridae*. VEEV is endemic throughout Central and South America, circulating in rodent and/or avian hosts and a mosquito vector (Weaver *et al.*, 2004). An epidemic in Colombia in 1995 resulted in an estimated number between 75 000 and 100 000 human cases, of which 3000 had neurological complications and 300 were fatal (Rivas *et al.*, 1997; Weaver *et al.*, 2004). Clinical signs in humans include fever, severe headache, nausea, photophobia, viremia, and lymphopenia (Weaver *et al.*, 2004). The mortality rate in equines during epizootics is estimated to be around 19–83%, and less frequently in humans, with neurological diseases appearing in 4–14% of the cases. VEE occurs in all age groups, and fatal encephalitis is more likely to develop in children (Weaver *et al.*, 2004). Before the introduction of vaccination against VEE for at-risk personnel, VEEV infection was documented among 150 laboratory personnel, with one fatality (Smith *et al.*, 1997). VEEV is highly infectious as an aerosol, and has therefore been studied in the past for use as a biological weapon (Smith *et al.*, 1997).

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14. ABSTRACT

Host responses to Venezuelan equine encephalitis viruses (VEEV) were studied in cynomolgus macaques after aerosol exposure to the epizootic virus. Changes in global gene expression were assessed for the brain, lungs, and spleen. In the brain, major histocompatibility complex (MHC) class I transcripts were induced, while the expression of S100b, a factor associated with brain injury, was inhibited, as was expression of the encephalitogenic gene MOG. Cytokine-mediated signals were affected by infection, including those involving IFN-mediated antiviral activity (IRF-7, OAS, and Mx transcripts), and the increased transcription of caspases. Induction of a few immunologically relevant genes (e.g. IFITM1 and STAT1) was common to all tested tissues. Herein, both tissue-specific and nontissue specific transcriptional changes in response to VEEV are described, including induction of IFN-regulated transcripts and cytokine-induced apoptotic factors, in addition to cellular factors in the brain that may be descriptive of the health status of the brain during the infectious process. Altogether, this work provides novel information on common and tissue-specific host responses against VEEV in a nonhuman primate model of aerosol exposure.

15. SUBJECT TERMS

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Because aerosol transmission is not the natural route of VEEV infection, it represents a unique situation. Previous studies in mice and rhesus macaques (*Macaca mulatta*) showed that after s.c. inoculation mimicking a mosquito bite, VEEV infects lymphoid tissues, circulates in the blood, and may enter the brain through the olfactory nerve (Gleiser *et al*., 1962; Vogel *et al*., 1996). VEEV aerosol infection is thought to bypass the viremia before infection of the olfactory bulb. In experimental animals, aerosol or intranasally delivered VEEV penetrates the brain 2–3 days earlier than is seen after s.c. inoculation (Danes *et al*., 1973a, b; Vogel *et al*., 1996; Steele *et al*., 1998). Cynomolgus macaques have been used as a model of the human disease caused by aerosol exposure to VEEV including efficacy studies for V3526, a VEEV vaccine candidate (Pratt *et al*., 1998; Reed *et al*., 2005). Macaques develop fever, lymphopenia, and signs indicating encephalitis after aerosol exposure to either epizootic or enzootic strains of VEEV (Pratt *et al*., 1998; Reed *et al*., 2004). However, clinical differences were noted in cynomolgus macaques when comparing between aerosol and s.c. exposure to VEEV (Pratt *et al*., 1998). S.c. infection induced a biphasic, high fever within the first 24 h of exposure, lasting for up to 6 days, when viremia, lymphopenia, anorexia, and depression became detectable. Aerosol infection induced a more prolonged course of the febrile state (appearing by 24 h but persisting longer, for at least 2 more days), and the animals exposed to aerosol presented more prominent lymphopenia.

VEEV is susceptible to IFN, and IFN resistance has been postulated to be a marker of epizootic potential (Jahrling *et al*., 1976; Spotts *et al*., 1998; White *et al*., 2001). However, not all studies agree with the notion that IFN resistance predicts VEEV virulence (Anishchenko *et al*., 2004). Because only a few reports are available on dissecting the host responses to VEEV infection, additional information on this subject is greatly needed. With this purpose in mind, this study investigated the changes in gene expression from various tissues of cynomolgus macaques after aerosol exposure to a virulent epizootic strain of VEEV.

Materials and methods

Animals

Healthy, adult cynomolgus macaques (*Macaca fascicularis*) were used from the nonhuman primate colony at the United States Army Medical Research Institute of Infectious Diseases (USAMRIID). Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council (1996). The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Aerosol-exposed monkeys were screened by enzyme-linked immunosorbent assay, and shown to be negative for previous exposure to VEEV, western, and eastern equine encephalitis viruses before assignment to these studies. All VEEV-exposed monkeys were males. The control group consisted of four monkeys, with one of them being a female. Quality analysis using the differentially modulated genes (the ratio of maximum to minimum signal intensity among the samples of ≥ 5) clearly showed that the female sample clustered together with the remaining control samples; hence, all the control samples were used in the data analysis (data not shown).

Virus

The Trinidad strain used in these studies is a virulent virus of the epizootic IA/B variety of VEEV and was originally isolated from the brain of a donkey. The viral stock used in this experiment had been initially passaged through adult guinea-pig once, chick embryo 14 times, suckling mouse brain twice, vero cells once, baby hampster kidney cells once, and finally vero cells once more. For aerosol exposures, virus was diluted to an appropriate concentration in Hank's-buffered saline solution containing 1% fetal bovine serum.

Aerosol exposure

Macaques were anesthetized by an i.m. injection of tiletamine/zolazepam (6 mg kg^{-1}), and a whole-body plethysmograph was performed to determine the animal's respiratory capacity. Subsequently, the animal was inserted into a Class III biological safety cabinet located inside a biosafety level-3 suite and exposed in a head-only aerosol chamber for 10 min to an aerosol generated by a Collison nebulizer as described previously at a concentration sufficient to achieve a presented dose of 1×10^8 PFU of VEEV (Reed *et al*., 2004).

Assessment of VEEV Infection in Blood

Hematology and differential blood tests were performed in the infected animals at intervals before (days -75, -60, -45, and/or -5) and after aerosol exposure (days $+2$, $+3$ or $+4$) (Table 1). At least three time points were measured to obtain the average for the preaerosol blood cell counts, and only on the euthanasia day for each specific group were the postexposure samples tested (Table 1). Viremia was also measured at days $+2$, $+3$, or $+4$ after exposure by performing a plaque-forming assay (Pratt *et al*., 1998).

RNA isolation and microarray hybridization

RNA was isolated from the brain, lungs, and spleen of two infected animals for each time point (days $+1$, $+2$, $+3$, and

Table 1. Effect on cynomolgus blood cell numbers after exposure to aerosolized VEEV

			Preexposure day						
Test	Group	Animal ID #	− 75	− 60	− 45	− 5	Mean	Postexposure	% change
Hematology									
WBC (K μL^{-1})	Day 2	**41–157**	5.4	5.6	3.6	4.3	4.7	1.9	− 59.6
		41–492	NA	6.4	7.4	8	7.3	2.5	− 65.6
	Day 3	**41–476**	8.5	12.1	8.5	9.8	9.7	3.8	− 60.2
		41–479	6	10	5.2	9.9	7.8	5	− 35.9
Normal range (4.0–15.0 K μL^{-1})	Day 4	91–494	7.3	9.3	5.3	12.6	8.6	7.7	− 10.5
		91–498	6.8	6.8	4.7	12.4	7.7	6.4	− 16.9
Differential									
Absolute lymphocytes (K μL^{-1})	Day 2	**41–157**	1.43	1.47	1.63	1.8	1.6	0.52	− 67.5
		41–492	NA	0.47	2.63	3.8	2.3	0.9	− 60.9
	Day 3	**41–476**	2.42	2.44	1.23	2.9	2.2	1.4	− 36.4
		41–479	2.3	1.84	1.39	6.6	3	1.7	− 43.3
Normal range (1.0–5.0 K μL^{-1})	Day 4	91–494	3.84	3.98	2.15	6.3	4.1	3.1	− 24.4
		91–498	2.22	1.82	1.27	3.4	2.2	1.5	− 31.8
Absolute monocytes (K μL^{-1})	Day 2	**41–157**	0.56	0.62	0.28	0.5	0.5	0.2	− 60
		41–492	NA	0.54	0.59	0.8	0.6	0.2	− 66.7
	Day 3	**41–476**	0.65	1.28	0.46	0.3	0.7	0.3	− 57.1
		41–479	0.32	0.39	0.06	0.6	0.3	0.4	33.3
Normal range (0.0–1.0 K μL^{-1})	Day 4	91–494	0.44	0.48	0.25	1.1	0.6	0.4	− 33.3
		91–498	0.17	0.28	0.2	0.6	0.3	0.1	− 66.7

Bold indicates animals with significant changes (> 35% difference between preexposure and postexposure values).
Shaded areas indicates significant change occurs in both animals within time point.

Table 2. DNA primer sequences for real-time RT-PCR reactions

Affymetrix annotation	Gene symbol	Sense primer	Antisense primer
AFFX-HUMGAPDH/M33197_3_at	GAPDH	5′-GAAGGTGAAGGTCGGAGTC-3′	5′-GAAGATGGTGATGGGATTTC-3′
205989_s_at	MOG	5′-GAATCTTTCCTTCCTCTCATCC-3′	5′-GTTTGTTTGTGTCTGACCATC-3′
208146_s_at	CPVL	5′-TGGAAAGGATCCCAGGAATAC-3′	5′-TCCTCCACCTCGAATAATTACC-3′
202269_x_at	GBP1	5′-AGACGACGAAAGGCATGTAC-3′	5′-TAGTGACGCTTGTTCCAAATTC-3′

+4, except that no spleen RNA was used for day +1). RNA was also harvested from unexposed control animals (spleen RNA from three animals, and brain and lung RNA from four animals). Quality control (QC) of the RNA samples was performed using an Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA). Total RNA samples passing QC were subjected to target preparation for hybridization to the Affymetrix Human Genome U133 plus 2.0 Genechip arrays according to the manufacturer's specifications (Affymetrix Inc., Santa Clara, CA).

Primer design

Primers were designed using BEACON DESIGNER 4.0 (Premier Biosoft International, Palo Alto, CA) for selected genes identified in the Affymetrix gene expression analysis study (Table 2). Primers were ordered (Integrated DNA Technologies, Coralville, IA) and resuspended to a concentration of 20 μM with real time (RT)-PCR-grade water (Ambion Inc., Austin, TX).

Real-time reverse transcriptase-PCR

Total RNA (500 ng) was transcribed to cDNA using the Biorad I-script cDNA synthesis kit (Biorad, Hercules, CA) in a total volume of 20 μL. The resulting cDNA products were diluted 1 : 5 with water. Reactions consisted of 300 nM sense and antisense primers, 1 μL of diluted cDNA, and water to obtain a volume of 12.5 μL. SyBr-Green Supermix (Biorad, Hercules, CA) was added to obtain a final reaction volume of 25 μL. Reactions were run using an I-cycler IQ (Biorad, Hercules, CA) with the following parameters: 95 °C, 3 min (95 °C, 10 s; 56 °C, 15 s; 72 °C, 20 s) × 40 cycles for real-time data collection. Data were normalized to the reference gene GAPDH. The real-time RT-PCR data were expressed as the starting quantity (SQ) ratio between the gene of interest and

the reference transcript for GAPDH. SQ represents the SQ for a certain transcript. A decrease in gene expression would correspond to a decrease in the SQ ratio. Affymetrix microarray work and RT-PCR data were generated in the Core Laboratory Facility at The Virginia Bioinformatics Institute (Blacksburg, VA).

Microarray data import, normalization, and analysis

The data were imported from 33 Affymetrix CEL files using the robust multiarray average (RMA) algorithm (Irizarry *et al.*, 2003). Expression values were $\log_2$ transformed as part of the RMA normalization. To detect differential expression, the following linear mixed-model ANOVA was used to partition tissue, time, and animal variability from variability due to biological and experimental noise:

$$y_{gijk} = \mu_g + T_i + D_j + TD_{ij} + A(D)_{jk} + \varepsilon_{gijk}$$

where y_{gijk} is the expression of the gth gene for ith tissue, jth day, and kth animal. The estimate of micrograms gives the expression measure for a probe set of the gth gene. The symbols T, D, TD, and $A(D)$ represent effects due to tissue, day, tissue-by-day interaction, and animal-nested-within-day, respectively. The error for gene g for sample ijk is designated as ε_{gijk}. Tissue and day are fixed effects; animal is a random effect. Within this ANOVA model, linear contrasts were used to compare the brain RNA from the infected animals' brain (days +2, +3, and +4) with the baseline, brain control samples (day 0). Similar contrasts were performed for the lung and spleen. Day +1 lung and spleen samples were omitted from the contrast, as the GeneChip arrays for day +1 samples did not pass chip quality control check. The P-values for each condition were then corrected using a step-up false discovery rate (FDR) value of 10% (Benjamini & Hochberg, 1995) to produce a list of significantly differentially expressed genes for each contrast described above. These lists of statistically significant genes were further filtered to include only those genes that demonstrated two fold or greater up- or downregulation in at least one of the tissues tested. A number of these differentially expressed transcripts are shown in Table 3, annotated by the Probe ID and gene symbol given by Affymetrix. The P-values for the ANOVA model were calculated using log-transformed data. The data normalization and statistical analysis were performed using the assumption that the signal intensities were log-normally distributed. Note that the data were log transformed for ANOVA to transform a multiplicative effect into an additive effect. However, because the authors wished to interpret the magnitude of change as a ratio, the log-transformed data were inappropriate, as they had been converted from a multiplicative effect to an additive effect. Simply antilogging the mean of logged data does not produce the mean of the unlogged data; however, it does produce the geometric mean of the unlogged data. Antilogging a least squares (LS) mean produces a value that is called a 'LS geometric mean.' Thus, the fold changes represent the ratios of 'LS geometric means' between the two groups being contrasted (Partek software User Manual).

The principal component analysis (PCA) was performed on the data to identity the most global differences in gene expression among the different samples (Fig. 1). RMA, mixed-model ANOVA, FDR, PCA, and fold-change calculations were performed using PARTEK GENOMICS SUITE v6.0 software (Partek Inc., St Louis, MO). Gene ontology analysis was performed using ONTOEXPRESS (Draghici *et al.*, 2003). Requests for the microarray data should be directed to the corresponding author.

Results

Hematology of infected cynomolgus macaques

Hematology and differential blood tests were performed in the exposed macaques showing considerable changes in blood cell counts ($\geq$35% between the mean of the preexposure values and the postexposure value for the same animal). The two animals in the day 2 group, showed a decline in white blood cell (WBC) count (59.6% and 65.6% declines, respectively) as compared with the average baseline WBC counts taken for each animal before exposure to VEEV (Table 1). The WBC count was also reduced in the day 3 animals (a reduction of 60.2% and 35.9% for the two animals), but only minimally at day 4. In general, animals showing a decline in WBCs also showed some decrease in the absolute numbers of lymphocytes and/or monocytes in the blood. The virus titer in the blood was higher at days 2 and 3 (data not shown), which is when a greater decrease in WBC count was noted (Table 1).

Global gene expression profile of VEEV-exposed cynomolgus macaques

Microarray analysis allowed evaluation of possible cellular mechanisms that may be associated with inhalation of infectious VEEV. A total of 33 samples were hybridized to arrays; however, five samples were omitted as outliers. The outlier chips showed unusual distribution for their expression levels as compared with the other 28 microarray chips. At least three of the five outlier chips contained low amounts of cRNA, which might help explain their poor hybridization. The remaining two outliers also appeared to have poor hybridizations as determined by visual inspection of the arrays, and their distributions were substantially different from the remaining 28 samples, even though they had adequate amounts of cRNA. After removing the five outlier

Table 3. Gene ontology classification and tissue expression ratios after VEEV aerosol exposure

Gene Symbol	Affymetrix probe ID	*P*-value (brain)	Brain	*P*-value (lung)	Lung	*P*-value (spleen)	Spleen
Antigen processing, endogenous antigen via MHC class I (BP)							
HLA-A	213932_x_at	**8.58E-05**	**2.40**	9.94E-02	1.22	9.42E-02	1.30
HLA-B	211911_x_at	**1.18E-03**	**2.70**	5.02E-02	1.50	2.94E-01	1.30
HLA-C	216526_x_at	**6.47E-04**	**2.58**	5.98E-02	1.40	1.52E-01	1.37
HLA-E	217456_x_at	**8.55E-04**	**2.63**	3.69E-02	1.51	1.16E-01	1.46
HLA-F	221875_x_at	**1.09E-03**	**2.20**	3.68E-03	1.75	1.59E-01	1.32
HLA-G	211530_x_at	**6.38E-05**	**2.31**	1.36E-02	1.36	1.83E-01	1.20
Antigen processing, exogenous antigen via MHC class II (BP)							
HLA-DQB1	211656_x_at	9.62E-01	1.01	2.04E-01	1.15	**1.69E-04**	**−2.33**
Apoptosis (BP)							
CASP1	211367_s_at	4.69E-01	1.11	**4.22E-05**	**2.57**	2.77E-01	−1.20
CASP4	209310_s_at	3.10E-02	1.38	**5.45E-05**	**2.30**	1.24E-02	1.57
Cell proliferation (BP)							
IL15	205992_s_at	1.70E-01	−1.33	**1.27E-03**	**2.22**	9.27E-01	−1.02
ISG20	204698_at	3.92E-01	1.26	**4.65E-04**	**3.54**	4.79E-03	3.08
S100B	209686_at	**8.87E-05**	**−3.91**	8.29E-01	1.04	8.38E-01	1.05
Inflammatory response (BP)							
IL1RN	212657_s_at	1.50E-01	1.35	2.94E-02	1.55	**7.78E-04**	**3.10**
IRF7	208436_s_at	5.90E-02	1.52	1.76E-03	2.15	**7.26E-04**	**3.18**
CXCL11	211122_s_at	5.57E-01	1.24	**6.54E-04**	**5.32**	6.56E-03	4.38
CCL3	205114_s_at	3.36E-01	−1.13	3.77E-03	1.51	**8.27E-04**	**2.01**
TNFAIP6	206025_s_at	3.75E-01	−1.09	4.23E-02	1.20	**4.33E-06**	**2.97**
Immune response (BP)							
OAS1	205552_s_at	8.35E-02	1.76	1.26E-02	2.23	**1.00E-03**	**5.21**
OAS2	204972_at	2.51E-02	1.91	**6.19E-04**	**3.06**	5.15E-03	2.79
OASL	205660_at	4.03E-01	1.22	**5.08E-04**	**3.08**	**9.20E-04**	**3.82**
IL18	206295_at	7.95E-01	−1.02	2.19E-01	−1.10	**3.64E-07**	**−4.04**
GBP1	202270_at	5.44E-01	1.19	**5.10E-04**	**3.94**	4.38E-03	3.51
GBP2	202748_at	1.75E-02	2.06	**9.84E-05**	**4.53**	**4.09E-04**	**4.97**
IFITM1	201601_x_at	**2.93E-04**	**4.70**	**8.33E-04**	**3.17**	9.21E-03	2.69
IFITM3	212203_x_at	**2.12E-05**	**5.23**	3.30E-03	1.97	**2.14E-03**	**2.59**
MX1	202086_at	**1.58E-04**	**3.44**	**6.87E-04**	**2.38**	**1.83E-03**	**2.63**
MX2	204994_at	7.68E-01	1.06	**1.76E-04**	**3.05**	**1.09E-03**	**3.02**
STAT1	209969_s_at	**2.39E-05**	**4.87**	**2.99E-05**	**3.82**	**2.42E-05**	**6.07**
XCL2	214567_s_at	2.67E-01	1.14	**4.93E-06**	**2.72**	3.28E-02	1.37
Neurogenesis (BP)							
MOBP	242765_at	**1.39E-05**	**−3.86**	6.56E-01	1.06	6.49E-01	1.08
Nerve–nerve synaptic transmission (BP)							
MBP	209072_at	**2.46E-04**	**−3.04**	1.33E-01	1.30	7.91E-02	1.51
MOG	214650_x_at	**8.05E-07**	**−3.37**	3.32E-01	−1.08	3.58E-01	1.10
NCAM1	227394_at	3.81E-02	1.46	9.28E-02	1.29	**1.71E-04**	**−3.14**
Chemokine activity (MF)							
CKLF	219161_s_at	3.91E-01	−1.10	7.56E-03	1.38	**3.31E-05**	**−2.66**
XCL1	206366_x_at	5.81E-01	1.07	**3.04E-05**	**2.28**	4.66E-01	1.10
CXCL9	203915_at	4.97E-01	1.10	8.29E-03	1.52	**1.95E-03**	**2.04**
Transport (BP)							
TF	203400_s_at	**1.49E-06**	**−3.47**	5.99E-01	-1.05	2.16E-01	1.16
STX17	222708_s_at	5.58E-03	1.36	8.66E-03	1.28	**5.25E-05**	**2.08**

BP, biological process; MF, molecular function.
Bold values are significant.

chips, it was clear from the PCA that the most substantial global differences in gene expression were due to tissue type (Fig. 1). More specifically, the brain tissue appeared to be very different from the lungs and spleen [distinguished by principal component (PC) #1], and the lungs and spleen also had substantial differences (evident on PC #2). The mixed-model ANOVA was selected to model the effects of the experimental treatments of tissue, time, interaction of tissue, and time, and animal-to-animal variation. Based on this ANOVA model, contrasts of posttreatment time points (days 2,

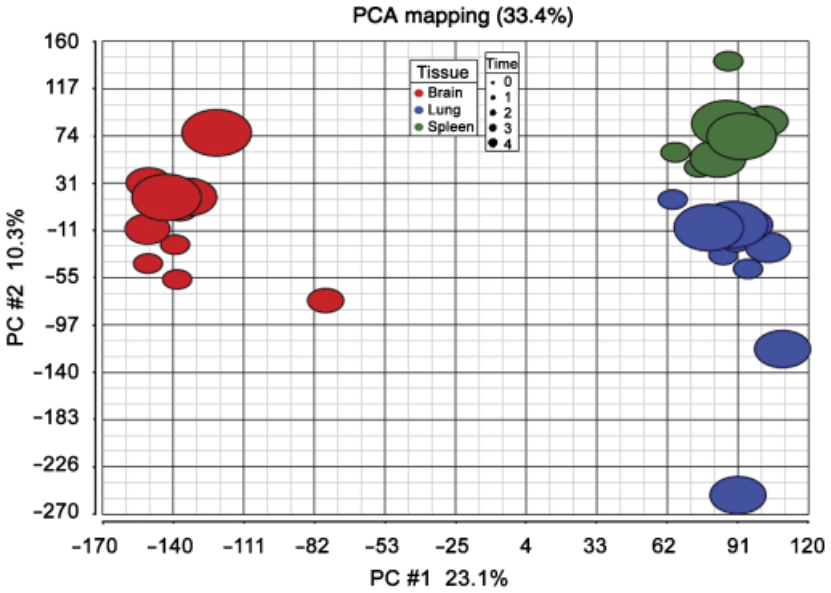

Fig. 1. PCA of the human chips hybridized to tissue RNA samples of VEEV-exposed cynomolgus macaques. PCA was performed on the microarray data to determine how the different samples clustered regarding tissue type and time of exposure to VEEV. The points are colored by tissue (brain: red; lungs: blue; spleen: green) and sized by time point (day). The largest differences are clearly due to the different tissues. The smallest dots in the plot are the day 0 samples (controls) and seem to be tightly clustered (low overall variance).

3, and 4) to baseline (day 0) were used to identify genes of specific interest in the brain, lungs, and spleen (i.e. 1280, 761, and 1207 significant Affymetrix Probe IDs/transcripts, respectively). When the list of significant genes was queried for genes that were differentially expressed at least twofold higher or lower than control levels in the brain, lungs, or spleen, 157, 129, and 309 transcripts (Affymetrix IDs) were detected, respectively. It would have been ideal if control animals had been subjected to a sham aerosol exposure for comparison purposes; however, the control animals were not exposed to an aerosol.

Then ONTO-EXPRESS (Draghici *et al.*, 2003) was used to further organize these genes into Gene-Ontology (GO) categories: biological process, cellular role, and molecular function. The biological processes or molecular functions, which are involved with these selected genes, include immune responses, cell proliferation, apoptosis, central nervous system responses, and intracellular transport (Table 3). Further investigation into the roles of these differentially regulated genes will help to establish their role in the infectious and/or immunological process during infection. VEEV exposure induced most prominently immune response genes (Table 3). From the GO results (Table 3), the IFN-induced transmembrane protein (IFITM) 1 and 2′, 5′-oligoadenylate synthetase (OAS) 1 are classic IFN-regulated antiviral proteins (Kim *et al.*, 2004), and were also found after VEEV exposure. Major histocompatibility complex (MHC) class I molecules (HLA-A, -B, -C, -E, -F, and -G) and a selected number of host genes having chemokine and inflammatory activities (e.g. CXCL11, CCL3, ILIRN, and IRF7) were also factors affected by VEEV infection (Table 3).

Figure 2 shows the time course of expression for relevant transcripts in the brain, lungs, and spleen obtained from the microarray data. The time-course plots display the LS means and the SE of the LS means from the ANOVA model and were produced using PARTEK GENOMICS SUITE v6.0.

Induction of the human leukocyte antigen (HLA)-A was seen in the brain at day 3 (Fig. 2a), with no effect in the lungs or spleen. A similar pattern of expression was shown when human probes for other MHC class I molecules were used (HLA, -B, -C, -E, -F, and -G) (data not shown). However, because macaques display major variations in the genomic organization of their HLA genes and the microarray probes were generated against human HLA molecules, one should refer simply to transcriptional changes in MHC class I or MHC class II genes in this study.

A decrease in the expression of transcripts for myelin oligodendrocyte glycoprotein (MOG) (Fig. 2c) and S100b (Fig. 2b) at days 1 and 2, respectively, was also observed in brain tissue. The expression of these transcripts remained low in the brain for the 4 days of the study. VEEV exposure also affected IL expression, including an increase in IL15 in the lungs at day 2 (Fig. 2d), and a reduction in IL18 levels in the spleen also at day 2 (Fig. 2e).

MHC class II molecules and related transcripts were also affected by VEEV. The expression of an MHC class II transcript (human probe set for HLA-DBQ1) was shown to have been decreased in the spleen at day 2, but it was not considerably affected in the brain or lungs (Fig. 2f). Transcripts for the carboxypeptidase, vitellogenic-like (CPVL) protein were reduced in the spleen at days 2, 3, and 4 (Fig. 2g). The signal transducer and activator of

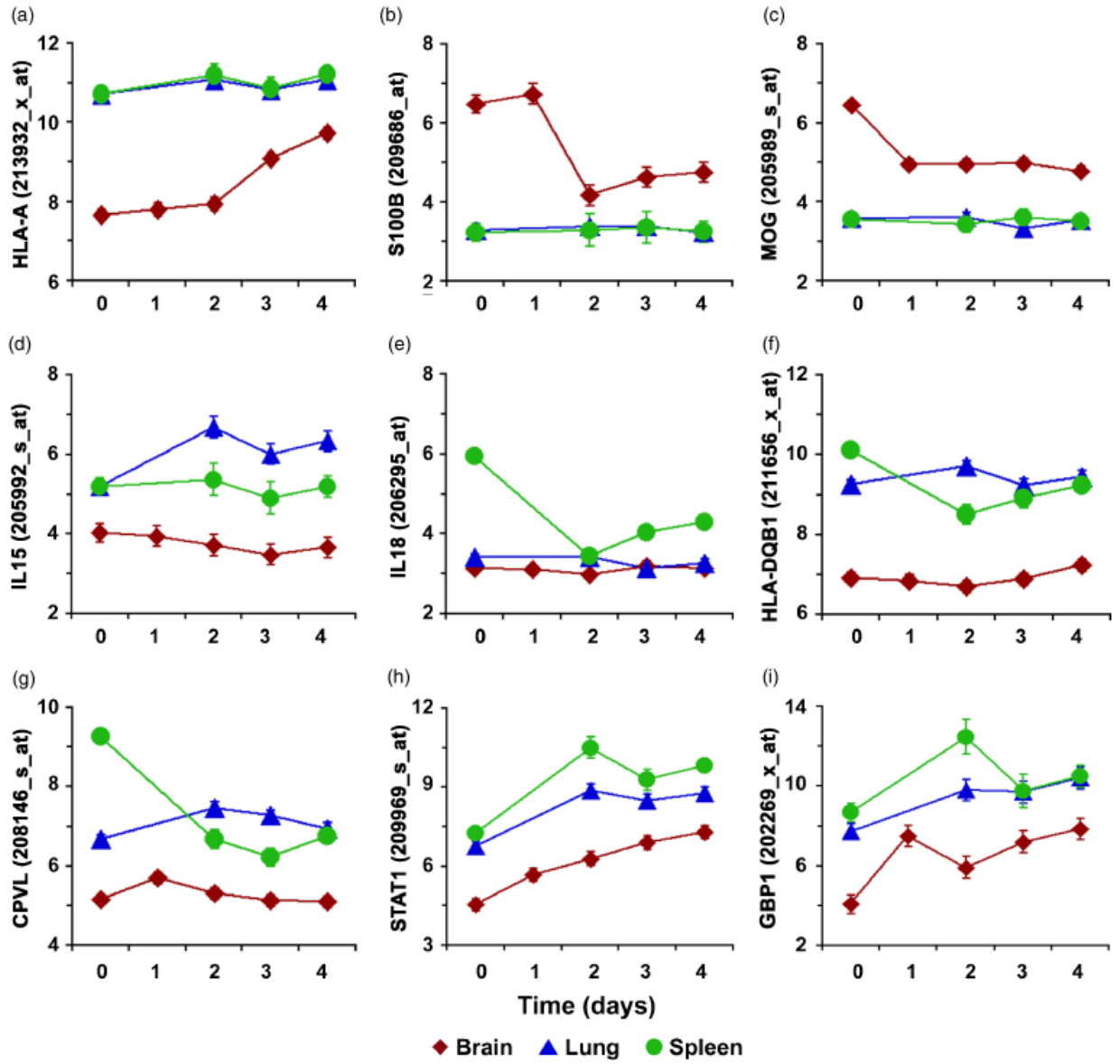

Fig. 2. Host transcriptional changes and tissue specificity of the response to aerosolized VEEV. (a) MHC class I genes such as HLA-A represented here were induced in the brain after day 2 postexposure. (b) S100b and (c) MOG were downregulated in the brain as early as day 2 and day 1, respectively. (d) Expression of IL15 in the lungs increased at day 2 and decreased slightly at day 3. (e) IL18 expression decreased at day 2 in the spleen; however, it did increase slightly afterwards. (f) Similarly, the levels of the MHC class II molecule HLA-DQB1 declined at day 2 in the spleen, and increased slightly afterwards, but remained unchanged in the brain and lung. (g) CPVL was also downregulated in the spleen. (h) Expression of the STAT1 transcript increased in all tissues, and (i) GBP1 expression peaked at day 2 in the spleen. Diamond = brain; triangle = lungs; circle = spleen. Time 0 correspond to unexposed cynomolgus macaques.

transcription (STAT) 1 was induced in all tissues tested in comparison with control (brain, lungs, and spleen, Fig. 2h). The transcript for guanylate-binding protein 1 (Fig. 2i), another immune-related gene, showed an increase in the spleen at day 2. A few apoptotic genes were affected by VEEV as well [caspase (CASP) 1, CASP 4, and S100b – Table 3].

Differential expression by real-time RT-PCR

The differential expression of CPVL initially observed in the microarray results was confirmed by RT-PCR. This serine carboxypeptidase is usually found in tissues with immune-related functions (Mahoney *et al.*, 2001). Little information is available on the possible roles of CPVL, except that CPVL

is involved in processing/transporting peptides for loading onto MHC class II molecules. The expression of CPVL declined in the spleen at day 2, and was slightly increased in the lungs at day 2 as well (Fig. 3a). Interestingly, the temporal expression profile for CPVL is similar to that for the MHC class II molecule (human probe set for HLA-DBQ1) (compare Fig. 2f with Figs 2g and 3a). The GBP-1 transcript peaked at day 2 in the spleen, and its expression was decreased again at day 3 postexposure (Fig. 3b). This response was also observed in the microarray results.

In addition, RT-PCR was performed for the MOG transcript. It was interesting that although MOG overexpression may lead to encephalitis (Mokhtarian *et al.*, 1999), the transcriptional levels of MOG were reduced by VEEV in the microarray data. Figure 3c shows by real-time RT-PCR that MOG was downregulated in the brain post VEEV exposure. In the microarray data, the pattern of expression for the brain was similar in all four MOG probe sets present in the chip (Fig. 2c and data not shown). A functional relationship between MOG downregulation and VEEV infection is yet to be established.

Discussion

Solid knowledge of the mechanisms of host response to VEEV infection can be very helpful for the prevention and emergency preparedness against an intentional aerosol exposure of the virus. VEEV will readily replicate in humans, as shown by past aerosol exposure incidents of laboratory personnel (Laboratory Safety, 1980). The aerosol route differs from the natural route of infection, and only a few reports have investigated the pathogenesis of aerosolized VEEV (Danes *et al.*, 1973a, b; Pratt *et al.*, 1998; Steele *et al.*, 1998; Reed *et al.*, 2004, 2005). Detailed pathogenesis and the host response to VEEV should be investigated, particularly with the tools available to modern biology and in nonhuman primates, the most relevant model of the human disease.

The present study investigated the changes in global gene expression during the course of VEEV infection. These changes included several genes from the Type I IFN signal cascade, and provided a basis to compare with host responses to other alphaviruses. Johnston *et al.* (2001), for example, investigated the infection of the Old World alphavirus Sindbis in murine brain, and compared the responses between a virulent and an avirulent strain of Sindbis (differing by a single amino-acid change). Both this study and the Sindbis study revealed a strong presence of IFN-regulated factors in the infection (compare Table 3 in this study with Johnston *et al.*, 2001), including STAT1, IRF-7, and the Mx1. In the brain, the IFN-regulated transcripts for IFITM-1 and IFITM-3 were upregulated by VEEV in this work, but not by Sindbis in Johnston's work. It is also intriguing that VEEV could be regulating the expression of a potentially encephalitogenic cellular factor (MOG), and the appearance of S100b, a potential marker for brain injury,

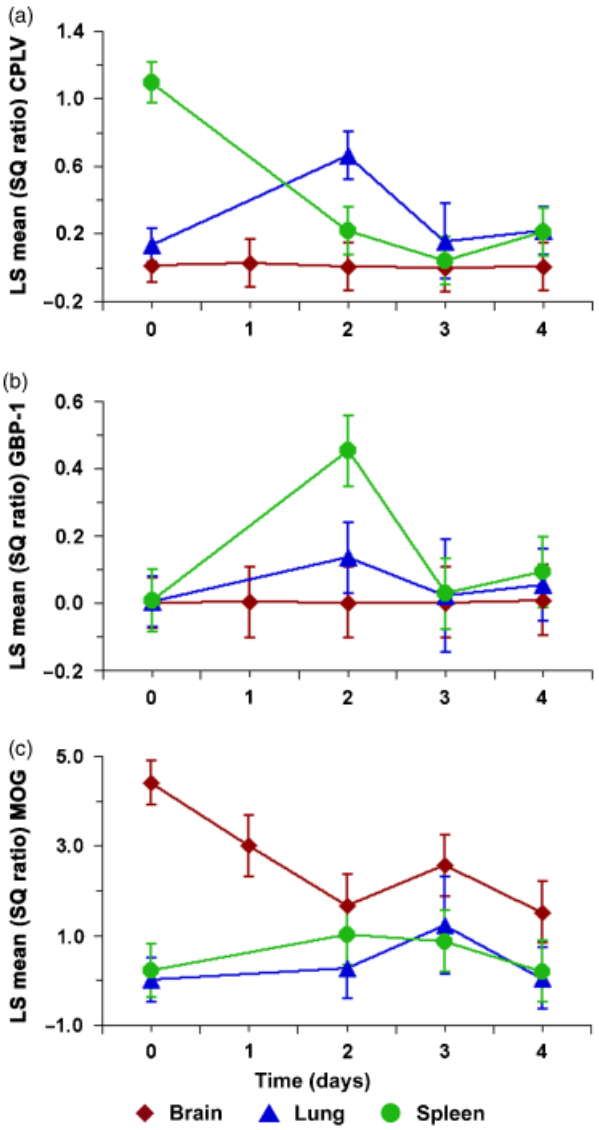

Fig. 3. Real-time RT-PCR analysis of VEEV-exposed tissues. The RT-PCR results for the transcripts for (A) CPVL, (B) GBP1, and (C) MOG are shown. The *Y*-axis represents the ratio between the SQ for the transcript of interest and that for the housekeeping gene GAPDH. (a) The SQ ratio for CPVL decreased with time in the spleen, indicating a reduction in CPVL transcripts. (b) GBP1 expression peaked at day 2 in the spleen. (c) MOG was downregulated in the brain as shown by a decrease in the SQ ratio (MOG/GAPDH) at day 2. MOG remains downregulated during the course of the study. The decrease in SQ corresponds to a decrease in gene expression. The SE is shown for each sample. Diamond = brain; triangle = lungs; circle = spleen. Two biological replicates and three technical replicates were used per condition, unless otherwise specified. Only one biological replicate was used for lung day 3. Time 0 represents the unexposed cynomolgus macaque data.

for its own benefit to survive in the host. The expression of both MOG and S100b was not reported to have changed in Sindbis infection (Johnston *et al.*, 2001). Comparison with additional alphaviruses will help build knowledge of the infectious process of alphaviruses and how that might differ from other infectious agents.

Induction of Mx1 and OAS transcripts (OAS1, OAS2, and OASL) in this work suggests the involvement of IFN-stimulated antiviral pathways (Haller *et al.*, 2006). Hamano *et al.* (2005) have already described the importance of OAS1 and MxA in viral infections, suggesting that polymorphisms in both genes affect the susceptibility and progression of severe acute respiratory syndrome (SARS). In addition to type I IFN responses, GBP-1, a factor induced by IFNγ, was also upregulated by VEEV (Anderson *et al.*, 1999). Other factors induced by inflammatory cytokines, IFITM1 and IFITM3, were also induced, and this was common to all the tissues tested (brain, lung, and spleen). STAT1, another cellular factor induced by IFN and other inflammatory cytokines, had a similar pattern of expression. STAT1 transcript is induced by other neurotropic viruses, such as Sindbis and the Japanese encephalitis virus from the *Flaviviridae* family (Johnston *et al.*, 2001; Saha & Rangarajan, 2003).

The increase in MHC class I transcripts by VEEV (Fig. 2, Table 3) correlates with what is seen for other alphaviruses in the brain (Kimura & Griffin, 2000). This increase may promote clearance of the infected cells or may perhaps affect cytokine-induced $CD8^+$ T cell clearance of viral transcipts (Kimura & Griffin, 2000). MHC class I molecules have also been identified as cell surface receptors for alphaviruses, as has been shown for HLA-A and -B molecules regarding SFV (Helenius *et al.*, 1978). In the case of VEEV infection, the role of MHC class I molecules awaits further evaluation.

The IL-18 precursor is cleaved and activated by caspase 1 (Hodges *et al.*, 2001). Although IL-18 expression was reduced in the spleen at day 2 (Fig. 2e), caspase 1 did not change (Table 3). Reduction in IL-18 may impair IFN activity (Hodges *et al.*, 2001), and cytotoxic T-cell responses. Interestingly, CPVL, a factor that may play a role in processing and/or transport of MHC class I peptides, or in MHC class II APC functions (Harris *et al.*, 2006), was also downregulated at day 2 in the spleen. At day 2, the transcript for the MHC class II molecule HLA-DBQ1 was also reduced in the spleen, suggesting inhibition of both MHC class I and class II responses. In the lungs, on the other hand, an increase in cytokine levels (IL-15), and in caspase 1 was observed. This increase may reflect the early signs of a Th-1 response (Muro *et al.*, 2001; Zuo *et al.*, 2005). Furthermore, the presence of the cytokine activation group of caspases, CASP 1 and CASP 4, in the lungs suggests that apoptosis in the infected lung is at least in part a cytokine-mediated event.

The authors' laboratory and others have previously described a reduction in peripheral blood leukocytes in cynomolgus macaques infected via aerosol exposure to VEEV (Pratt *et al.*, 1998; Reed *et al.*, 2004). Similarly, in the present study, immune cells (WBC) in the blood of the infected macaques were considerably reduced at days 2 and 3 postinfection (Table 1). In these groups, a greater reduction in circulating lymphocytes and/or monocytes, and higher viral titers were also noted (Table 1 and data not shown). Interestingly, it was also between days 2 and 3 postinfection that a decrease in transcripts for IFN-regulated genes was observed in the spleen [OAS, Mx, IFITM (data not shown), and GBP genes (Fig. 2i)]. This result may reflect, at least in part, an attempt by the virus to evade the immune response by suppressing the IFN response between those time points. It is the identification of specific factors participating in critical immune-related functions, such as the type 1 IFN responses (e.g. STAT1, Mx1, and OAS transcripts, IRF-7) (Haller *et al.*, 2006), and IFNγ-regulated responses (GBPs and IFITMs) (Lubeseder-Murtellato *et al.*, 2002) that will help visualize the strategy for host defense.

The brain showed inhibition of the MOG transcript. The 28-kDa MOG glycoprotein accounts for *c.* 0.1% of all CNS myelin in humans (Delarasse *et al.*, 2003), and its overexpression may cause autoimmune encephalitis. Semliki Forest Virus (SFV), another alphavirus, expresses a structural protein with an amino-acid sequence (E2 peptide 115–129) with potential molecular mimicry to the host MOG peptide 18–32 (Mokhtarian *et al.*, 1999; Parades *et al.*, 2001). This SFV peptide has been shown to induce a later-onset chronic experimental autoimmune encephalomyelitis, with a pathology resembling secondary demylelination occurring in SFV infections (Mokhtarian *et al.*, 1999). The reason for the inhibition of the MOG transcript by VEEV may not be related to molecular mimicry but it should be investigated. The S100b transcript was also inhibited by VEEV in the brain. This factor has been previously associated with brain injury, and could serve as a sensor for brain injury. Additionally, S100b is known to affect p53-response and/or neuronal cell survival or proliferation (Arcuri *et al.*, 2005).

During analysis of the data, it was considered that microarray data interpretation from nonhuman primate samples hybridized to human gene chips should be made with a degree of caution; however, a number of publications have dealt with this issue and shown that testing of nonhuman primate RNA using human arrays is acceptable (Ace and Okulicz, 2004; Rubins *et al.*, 2004; Wang *et al.*, 2004; Dillman III *et al.*, 2005). The authors are also aware that even minute differences in gene expression can induce dramatic phenotypic changes. For example, in the case of Down syndrome, an individual carrying an extra copy of chromosome 21 has an overall difference in gene expression

of 1.37-fold over control (Mao *et al*., 2005). Minute changes in host gene expression should be considered as they are also expected to impact VEEV infection and immunity.

In the present study, a list of differentially expressed genes was compiled. Some of these genes showed a tissue-specific pattern of expression (brain, lung, or spleen), and some had similar profiles regardless of tissue type (e.g. IFITM and STAT1) (Table 3). Now that the patterns of gene expression are known for aerosolized VEEV infection in nonhuman primates, it would be interesting to compare the data with the results of an s.c. infection. Fever develops slightly earlier in cynomolgus macaques infected by the s.c. route; however, it will last longer in animals exposed by aerosol (Pratt *et al*., 1998). This result may imply that endogenous pyrogens occur slightly earlier in s.c. infections, but are more prolonged by aerosol. Lymphopenia also seems to be more aggressive in aerosol infection; therefore, expression of apoptotic (e.g. CASP 1, CASP 4) and proinflammatory genes (e.g. IFN-regulated genes) may be more potent and/or prolonged in animals exposed to VEEV by the aerosol route. Accumulation of tissue-specific and route-specific host response data is critical to provide the foundation for the understanding of disease progression, host defense mechanisms, and identification of VEEV biomarkers.

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